BIOCHEMICAL VARIATIONS DURING CALLUS DIFFERENTIATION , REGENERATION POTENTIAL OF CALLUS OF *LAWSONIA INERMIS* L. UNDER *IN VITRO* CONDITIONS AND ESTIMATION , STANDARDIZATION METHOD TLC PLATE SECREENING FOLLOWED BY SOLID PHASE EXTRACTION OF LAWSONE COMPOUND USING THROUGH UPLC

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Abstract :
Lawsonia inermis commonly known as Mehandi or Henna , belongs to family Lythraceae .The present work was carried out with the objectives to devise suitable media for culture initiation , indirect organogenesis and biochemical estimations of callus induction during differentiation . Murashige and Skoog (1962) medium was used for the present investigation .This medium was fortified with various amount of auxins like Indole acetic acid , Napthalene acetic acid , 2,4-Dichlorophenoxy acetic acid (0.5,1.0,1.5,2.0 and 3.0 mg/l) and cytokinins like Kinetin and Benzylamino purine (0.5,1.0,1.5,2.0 and 3.0mg/l) individually and in various combinations .The medium containing , Dichlorophenoxy acetic acid resulted in highest percent induction of callus .There was no callus induction from internodal segments on medium devoid of growth regulators. Maximum percent callus induction upon cytokinin supplemented media was on BAP (3.0). The higher growth value callus developed from internodal segments was used for biochemical studies .There was decline in the contents of starch whereas the total soluble sugars , reducing sugars and total soluble proteins got increased .The activity of the enzyme alpha amylase of callus got increased and the activity of other enzymes *i e.* acid invertase is and acid peroxidase got decreased with the passage of time . UPLC analysis of extracts revealed that lawsone (naphthoquinone) was found in mother plant , regenerated plants and calli derived from intermodal segments of *Lawsonia inermis*. Highest lawsone content yield (0.154 µg /mg) was observed in leaves of plant tissue culture raised plants with as compared to mother plant and the calli.

Key words:- Biochemicals , Callus , Indirect organogenesis, regeneration , TLC plate , Soild phase extraction , UPLC, Lawsone

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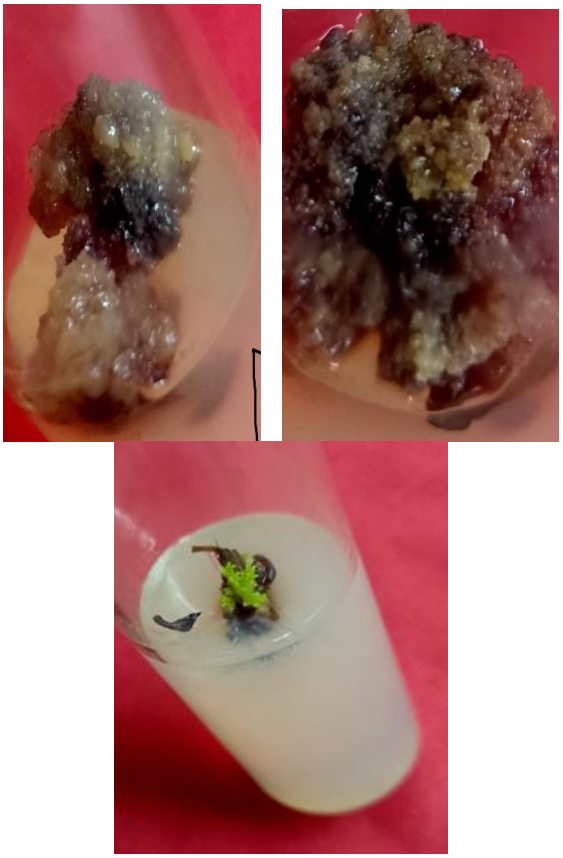
Introduction :

The demand for medicinal plant based raw materials is growing at a faster rate .The genetic diversity of medicinal plants in the world is getting endangered because of over-harvesting for production of medicines , habitat degradation , industrialization , illegal trade practices , loss of regeneration potential of degraded forests. All these factor are responsible for the loss to the biodiversity. Micropropagation methods help in solving such type of problems .This is an important tool for the *ex-situ* conservation of elite, rare , endangered and economically important plant species. It has an edge over traditional techniques of plant multiplication for a fast and mass multiplication of plants under *in vitro* conditions .These techniques are being used globally for the multiplication and conservation plant biodiversity. *Lawsonia inermis* L. commonly known as Mehandi or Henna , selected for the present investigation , belongs to family Lythraceae . It is a dwarf shrub and is well-known worldwide for its cosmetic and medicinal uses . It contains a large number of bioactive compounds which make this plant as a rich resource for various medicines. This plant has analgesic, hypoglycemic, heptatoprotective,immunostimulant,antiinflammatory,antibacterial,antimicrobial,antifungal,antiviral ,antiprasitic,antitrypanosomal , antidermatphytic , antioxidant , antifertiliy , tuberculosis and anticancer properties . It is also popular among women for colouring the hairs and hands in artstic way. Due to the economic , medicinal and pharmaceutical importance of this plant species , the present work was carried out with the objectives to devise suitable media for culture initiation , indirect organogenesis and biochemical estimations of callus during differentiation . Effect of various plant growth regulators and additives have been documented . Attempts have also been made for estimation of lawsone content from leaves and callus using solid phase extraction followed by UPLC technique.

Materials and Methods-

For indirect organogenesis , MS medium with different concentrations of growth regulators like IAA , α-naphthalene acetic acid , 6-benzylaminopurine, kinetin and sucrose (3%) were used .The pH of the medium was regulated 5.8. After three days, the medium was used for inoculation of internodal explants. Internodal explants (1.0- 1.5cm) taken from mother plant of *L. inermis* growing in Herbal garden , Department of Botany, Kurukshetra University, Kurukshetra were inoculated on the Murashige and Skoog (1962) medium by using aseptic conditions .The explants were inoculated on MS medium supplemented with different concentrations (0.5-3.0 mg l-1) of cytokinins (BA and Kn) and auxins (IAA, NAA, 2, 4-D, IBA and TDZ) alone and in different combinations for callus induction and proliferation in plant tissue culture tubes .These were incubated at a particular temperature 25±2º C, illuminating light photoperiod of 16 hour light and 8 hour dark with a photosynthetic photon flux density of 40 µ mol m^{-2} s^{-1}. Various metabolites and enzymes like starch (Hassid and Neufeld, 1964) , total soluble sugars (Yemm and Willis,1954) , reducing sugars (Handa *et al*. ,1982), total phenols (Amorim *et al*. (1977) , nitrogen (A.O.A.C, 1970) , potassium (flame photometer), phosphorus (Jackson ,1973), α - amylase (Shuster and Gifford ,1962), acid invertase (Summer ,1935), acid peroxidase (Seevers *et al*. , 1971) were estimated from callus. Lawsone contents of mother plant , regenerated plants and callus were analysed solid phase extraction followed by using UPLC .



Callus diagram shown figure – (A) (B) (C)

Initiation of Aseptic Cultures -

Excised internodal segments from mother plant of *Lawsonia inermis* were treated with different sterilizing agents for variable durations and inoculated on agar solidified MS medium . Data

were recorded for viability rate and contamination percentage of explants after 3-4 weeks of inoculation .Various types of sterilizing agents were used for different intervals of time to surface sterilize the internodal explants (Table 1). The frequency of survival explants varied with concentration of Bavistin , Streptocyclin , $HgCl_2$ and sterilization time . Highest percentage (100%) of survival rate and asepsis was observed at treatment (0.2% Bavistin and 0.2% Streptocyclin for 90 min + 0.1% $HgCl_2$ for 3 min) followed by different concentrations of mercuric chloride (0.1% $HgCl_2$ for 3 min + 70% ethanol for 30 sec + 0.2% Bavistin for 90 min and 0.2% Streptocyclin for 60 min) treatment with (3.7%) microbial contamination . It was observed that when the 0.1% $HgCl_2$ alone for 1 min is used as sterilitant , maximum percentage (81.5%) of microbial contamination observed but it was lower than control treatment on which highest percentage (100%) of contamination was observed .The highest percentage (100%) of asepsis and survival rate of internodal explants was observed at treatment (0.2% Bavistin and 0.2% Streptocyclin for 90 min + 0.1% $HgCl_2$ for 2.5 min) followed by treatment (0.2% Bavistin for 90 min and 0.2% Streptocyclin for 60 min + 0.1% $HgCl_2$ for 2.5 min) with (7.4%) microbial contamination. Aseptic environment is the necessary step for settlement of healthy cultures under *in vitro* conditions. Contamination is a big problem in maintaining the cultures of the plants (Sarasan *et al,* 2006). Field collected explants are extremely prone to microbial contamination , therefore, it is initial most step to create a healthy, microbe free environment (Yadav *et al*, 2014). It is necessary to standardize the sterilization protocol to explants without harming the tissue (Chawla , 2003, Verma *et al*, 2012). For *in vitro* cultures , $HgCl_2$ was proved to be more effective as compared to other chemicals for various plants (Nagesh, 2008, Sharanappa and Rai, 2011). Higher concentration of $HgCl_2$ with prolonged time showed side effects on the health of explants (Danso *et al*, 2011). It was also reported to be successful sterilitant which was found in *Zea mays* (Huang and Wi , 2004), *Calophyllum brasiliense* (Silveria *et al*, 2016) , *Hylocereus polyrhizus* (Qin *et al*, 2017) and *Persea bombycina* (Boruah, 2020).

Indirect organogenesis

Internodal segments for the purpose of indirect organogenesis were inoculated on medium having various growth regulators singly or in combinations. There was no callus induction from internodal segments on medium devoid of growth regulators . Addition of growth hormones recorded in callus induction but the responses varied with the kind and strength of growth hormones. Callus started from the cut surface of the plant parts and lastly whole of the surface of the explants was involved . The medium with 0.5 Kinetin and 1.0 Kinetin supplemented alone failed to induce callus . Higher concentration of kinetin resulted in the callus formation (Table-2). Maximum percent callus induction upon cytokinin supplemented media was on BAP (3.0). The number of days taken for callus induction was also less in this medium .The callus growth was also moderate on this medium but the growth of callus was poor on the other cytokinin supplied medium. Fortification of auxins to the media resulted in moderate to good callus growth (Table-2). Among the auxins supplemented media , medium containing 2, 4-Dichlorophenoxy acetic acid resulted in highest percent induction of callus .This medium also supported very good growth of callus .The callus was greenish white in colour. Combinations of BAP with Kinetin and auxins were also tried for the induction and growth of callus. Combining BAP with kinetin could not improve the percent callus induction but it was better than the individually given kinetin. The growth of the callus was also poor. Addition of auxins in place of kinetin with BAP, proved better in terms of callus induction and growth. Highest percent of callus induction and very good growth of callus from the internodal segments were observed on medium with BAP (3.0) + (2.0) 2,4-D. Percent callus induction increased with the increase in concentration from 0.5 - 2.0 and further enhancement in strength resulted in decrease in the percent response (Table 3).The calli were sub-cultured also on the regenerating media to study the organogenetic potential .The callus cultured on medium without growth regulators failed to show any growth as well as organogenesis.The medium given with BAP (2.0) resulted in regeneration of shoots from the callus after 20 days of sub-culturing . Other cytokinins given media were not able to produce shoots or roots. To achieve micropropagation through callus , it requires the induction and formation of callus as an initial step. In the present investigation, internodal segments were made to induce callus on MS medium added with cytokinins & auxins individually as well as in amalgamation . It was observed that the medium devoid of growth regulators was not able to initiate callus from the tested explants which is probably because of the insufficient level of endogenous growth hormone in explants to induce callus and it required an outside supply. Initiation of callus took

place with the application of growth regulators .Callus initiated from the cut ends of the explants and finally whole surface of the explants was involved. Similar type of findings have been noted in hypocotyls and cotyledonary explants of *Leucaena leucocephala* (Singh and Lal , 2007) and *Sesbania grandiflora* (Khattar and Mohan Ram, 1983). This may be because of the formation of internal auxins from the damaged cell of cut surface which enhanced the cell division as observed in *Ornithogallum (*Hussey, 1976) where active cell division noticed at cut ends of the tissue. But in present study, the auxins released by the damaged tissue were not sufficient to trigger cell division and it required exogenous supply of auxins . Among the auxins, 2,4-dichlorophenoxy acetic acid was recorded to be better in respect to per cent callus initiation and time period required for initiation and growth of calli . The fortification of auxins and cytokinins together in variable concentrations produce better results in terms of callus induction and growth . Similar findings have been observed in *Asparagus officinalis , Coscinium fenestratum* and *Elaeagnus angustifolia* by Khan *et al*, 2008, Zeng *et al*, 2009 and Ha *et al*, 2008 respectively. The action of cytokinins and auxins in callus initiation was also reported by Yasmeen and Rao, 2005 , Latto *et al*, 2006 , Juniad *et al* 2007 and Barbulis *et al* , 2008 in moong bean,, *Chlorophytum arundinacem* , *Catharanthus roseus* and *Brassica napus*. It was concluded that the plant parts used for aseptic culture, need an ideal quantity of growth hormones for their differentiation in to unorganized callus.

Biochemical and Enzymes –

The higher growth value callus developed from internodal segments was used for biochemical studies. Various metabolites like total soluble sugars, starch , total phenols and total soluble proteins were estimated at 0^{th}, 5^{th} , 10^{th}, 15^{th}, 20^{th} and 25^{th} days after inoculation of callus . The callus was harvested on 0^{th} , 5^{th},10^{th}, 15^{th}, 20^{th} and 25^{th} days after inoculation .Initially, the starch contents were high and it got declined linearly with the period of sub culturing. The starch contents were highest (35.3 ± 1.3 mg/g dry wt.) at control and minimum (24.3 ± 1.5) at 25^{th} day after inoculation (Table 4). A steady increase in the total soluble sugars contents were noticed after sub culturing. It was highest (68.1 ± 1.5) at 25^{th} day after inoculation and lowest (38.2 ± 1.2) at control . Reducing sugars showed a decline trend up to 15^{th} day but after this showed a slight increase upto 25^{th} day of inoculation .The reducing sugars contents were 20.3 ± 1.3 at control and 12.3 ± 1.4 at 15^{th} day after inoculation. The total phenol contents of the callus were noticed to increase after the passage of time. These were minimum (53.3 ± 2.4) at the initial stage and maximum (66.1 ± 2.8) at 25^{th} day after culture .The total soluble proteins contents of callus were higher (4.4 ± 0.4) at 25^{th} days after transfer and minimum (2.1 ± 0.3) at 0^{th} days of transfer. There was a steady rise in protein contents of callus.The activity of the enzyme amylase of callus got increased with the passage of time . It was more (25.3 ± 1.5) at 25 days after inoculation and lesser (16.1 ± 1.3) at control (Table 5).The activity of other enzymes *i. e.* acid invertase and acid peroxidase got decreased with the passage of time. These were higher at initial stages and less at 25 days after inoculation (Table 4) . The value of acid invertase was minimum (2.32 ± 0.3b) at 25^{th} days and maximum at (4.50 ± 0.3) at control. Likewise, the value of acid phosphatase was minimum (2.25 ± 0.4) at 25^{th} days and maximum at (4.18 ± 0.5) at control .Differentiation *i..e.* the method of growth and morphophysiological specialization of cells from calli cells , is a requirement for the use of micropropagation for crop improvement .This process is regulated by phytohormones along with other constituents of culture medium (Smith and Krikorian , 1991). Some of the biochemicals and enzymes got changed during differentiation through callus (Sujatha *et al*, 2005, Dominic and Joseph, 2004). But very less is known about the biochemical changes happening in the cultured cells. Estimation of different cellular metabolites and enzyme activities gives a reasonable and promising approach to understand the biochemical basis of the development (Singh *et al*, 2009).To know the differentiation events in the callus tissues on resurgence medium , metabolic changes during organ differentiation in callus tissue were investigated . It was observed that starch content in callus decreased during the process of root and shoot development up to 25 days after sub culture . These findings coincides with the reports in *Nicotiana tobaccum* (Yadav *et al* , 1995, *Medicago arborea* (Martin *et al*, 2000, *Chlorophytum borivilianum* (Singh *et al*, 2006). This decline in starch contents may be because of the reduced activity of synthesizing enzymes or enhanced activity of hydrolyzing enzymes .The reduction in total soluble sugar content was associated with the use of sugars for growth and differentiation course. It was found that total soluble proteins in calli were more during differentiation than the control callus. As during differentiation , new proteins have to be manufactured because the cells are quantitatively changing their activities.Therefore, the protein

content is more before differentiation .Similar observations were also noticed by Yadav *et al*, 1995 , Mohapatra and Rath, 2005.Present investigation showed gradual reduction in phenolic contents with the passage of time. Phenols take part in the synthesis of cross- linking of cell wall constituents which is catalyzed by the enzyme peroxidase. Therefore, changes in the status of metabolites during shoot differentiation may be helpful in the understanding the biochemical basis of developmental pathway of this plant.

Mineral elements

Nitrogen, phosphorus and potassium were estimated in the mother plant, regenerated plants and the callus obtained from internodal segments . The data related to the elements contents is illustrated in Table 6. It was noticed that nitrogen contents were higher in regenerated plantlets as compared to mother plant and the callus. Likewise the phosphorus contents were also higher in the *in vitro* grown plantlets . Highest potassium contents were recorded in the formed plantlets in comparison with the parent plant and the callus. Minerals are the major constituents of micropropagation media .The requirement of minerals for a particular plant species is estimated by the changing of one or a combination of available formulations in literature. Generally, one nutritive medium is utilized for the whole time of culture, even then this composition may not be optimum for the various stages of explant growth and development .It is supposed that the mineral composition of the culture medium has a primarily helpful role in the resurgence process . Plants utilize these minerals as a component of structure in proteins and carbohydrates; metabolic organic molecules , enzyme activators and for maintaining the osmotic balance. In the present investigation, nitrogen, phosphorus and potassium were estimated in the mother plant, regenerated plants and the callus. It was recorded that nitrogen contents were higher in regenerated plantlets as compared to mother plant and the callus. Phosphorus and potassium contents were also higher in the regenerated plantlets. Higher levels of nitrogen, potassium and phosphorus may be due to better nutrition and performance under in vitro conditions.

Quantification of lawsone in mother plant, callus and regenerated plants methodology (SPE) -

Solid phase extraction method is a technique used to prepare samples via using a solid adsorbent present most commonly in a disk or on a cartridge device that is use to adsorb selected species from solution .This extraction methodology is generally utilized to cleansing- up the sample prior to be analyzed or for isolating a species from the sample. UPLC solid phase exraction analysis of extracts by using solid phase extraction revealed that lawsone was found in both mother plant and regenerated plants of *Lawsonia inermis*. Data presented in Table 7 showed that yield of lawsone content in mother plant leaves was 0.0281µg/ mg with 6834409 peak area at .748 retention time. Highest lawsone content yield (0.154 µg /mg) was observed in leaves of tissue culture raised plants with peak area 9084893 in 0.748 retention time . MS medium having 2.0 mg/l 2,4-dichlorophenoxy acetic acid was recorded to be best suited medium for initiation of calli and growth from internodal explants . This medium was taken as control to establish callus tissue from internodal explants to study the influence of various additives like Casein Hydrosylate , Adenine Sulphate and Yeast Extract on induction of lawsone (Table 8). UPLC estimation of lawsone from methanolic extract of callus derived from internodal segments under control resulted in 3411357 peak area with .748 retention time . Callus derived from internodal segments with different concentration of additives revealed that highest peak area 7996742 was on medium with 2,4-D 2.0 mg/ l + AdSO4 30 mg/l and with maximum yield 0.0824µg /mg lawsone content in .750 retention time followed by 0.0715 µg /mg lawsone content on medium with AdSO4 20 mg /l with 7260529 peak area and .749 retention time. All concentrations of yeast extract resulted in increased lawsone yield as compared to control . Addition of Casein Hydrosylate slightly affected the amount of lawsone. The stimulatory effect of elicitors was best shown in Adenine sulphate. Ultra performance liquid chromato-graphy (UPLC) has been used to quantify lawsone content in present study due to its high resolution , sensitivity, reproducibility and minimum requirement for extensive sample preparation . Lawsone (2-hydroxy-1-4- naphthoquinone) content of mother plant, regenerated plants and calli derived from internodal segments was estimated using this technique . Effect of MS medium fortified with 0.5 mg/ l 2,4-D containing different concentrations (10, 20, 30, mg/l) of various elicitors was studied for induction of lawsone (2-hydroxy -1-4nathoquinone) content in calli as depicted in Table 8. It revealed that all the elicitors used , have resulted in the increased amount of lawsone as compared to control .This may be due to biosynthesis of enzymes by these elicitors which are involved in the production of

secondary metabolites. Earlier also, methanol was noticed to be most suitable solvent to get higher yields for naphthoquinones extraction (Hu *et al*. 2006; Bernice *et al*. 2010, Wu *et al*. 2003; Marczak *et al*. 2005) likewise in the present investigation .Various chromatographic techniques (UPLC, HPLC, HPTLC) have been used to quantify secondary metabolites in medicinal plants by several workers (Dhiman *et al*. 2012 ; Gull *et al*, 2013 ; Netala *et al*. 2014) and their findings are in close agreement with the present study.The biosynthesis of secondary metabolite is genetically controlled but certain environmental and nutritional factor also play an important role (Vanisree *et al*. 2004).These metabolites accumulate in plants under stress conditions or in presence of various signal molecules or elicitors. Supply of elicitor has proved an effective way for improving the production of secondary metabolite in plant cell cultures. Putalun *et al* (2010) also reported that yeast extract increase the production of plumbagin in the roots of *D. burmanii* by 3.5 times as compared to control . Similarly, present investigation recorded that yeast extract enhances lawsone content 4-6 folds as compared to control.Whereas, reported there was 8.6 folds increase in lawsone content in cultures of Panichayupakaranant , Impatiens balsamina by the treatment with methyl jasmonate (Sakunphueak and 2010). In the present work, it was found that elicitors are able to induce lawsone content. This may be due to the activation of enzymes responsible for napthoquinone biosynthesis .

Table. 1. Effect of different durations of sterilizing agents on internodal segments.

S. No	Code	Bavistin Treatment		Streptocyclin Treatment		$HgCl_2$ Treatment (0.1%) conc.	Contamination percentage
		% conc.	Time (min)	% conc.	Time (min)	Time (min)	
1	T1	-	-	-	-	-	100
2	T2	-	-	-	-	0.5	81.5
3	T3	-	-	-	-	1.0	63.0
4	T4	-	-	-	-	1.5	55.6
5	T5	-	-	-	-	2	40.7
6	T6	-	-	-	-	2.5	37.0
7	T7					3 .5	33.3
8	T8	0.1	30	-	-	2.5	33.3
9	T9	0.1	60	-	-	2.5	29.6
10	T10	0.1	90	-	-	2.5	33.3
11	T11	0.2	60	-	-	2.5	18.5
12	T12	0.2	90	-	-	2.5	29.6
13	T13	0.2	120	-	-	2.5	22.2
14	T14	0.2	150	-	-	2.5	18.5
15	T15	0.2	120	0.1	60	2.5	18.5
16	T16	0.2	120	0.1	90	2.5	14.8
17	T17	0.2	120	0.2	60	2.5	14.8
18	T18	0.2	120	0.2	90	2.5	7.4
19	T19	0.2	120	0.2	120	2.5	0.0
20	T20	0.2	120	0.2	150	2.5	0.0

Table.2. Effect of auxins and cytokinins fortified individually on internodal segments of *L. inermis*

MS Media plus	Phytohormones (mg/l)	Period needed for callus initiation	Percentage of callus initiation	Callus texture	Growth of callus
MS control	-	-	-	-	-
KN	0.5	-	-	-	-
	1.0	-	-	-	-
	1.5	20	50	Milky, Greenish	+
	2.0	21	40	do	+
	2.5	21	50	do	+
	3.0	20	60	do	+
BAP	0.5	20	55	Milky, Greenish	+
	1.0	21	65	do	+
	1.5	21	50	do	+
	2.0	20	60	do	+
	2.5	20	60	do	+
	3.0	18	70	do	++
IAA	0.5	19	50	Milky, Greenish	++
	1.0	20	60	Milky, Greenish	++
	1.5	18	70	do	++
	2.0	20	60	do	++
	2.5	21	60	do	++
	3.0	20	60	do	++
NAA	0.5	20	55	Milky, Greenish	++
	1.0	20	60	do	++
	1.5	21	60	do	++
	2.0	19	60	do	++
	2.5	21	60	do	++
	3.0	20	60	do	++
2,4-D	0.5	19	60	Milky, Greenish	++
	1.0	20	60	do	+++
	1.5	19	55	do	+++
	2.0	18	80	do	++++++
	2.5	20	60	do	+++++
	3.0	19	60	do	++++

Table. 3 . Impact of auxins and BAP supplied jointly on internodal explants

MS Media plus	Growth regulators (mg/l)	Period needed for callus induction (days)	Percent callus initiation	Callus texture	Growth of callus
control	-	-	-	-	-
BAP(3.0)+ KN	0.5	20	61	Milky, Greenish	+
	1.0	21	59	do	+
	1.5	22	55	do	+
	2.0	21	56	do	+
	2.5	19	57	do	+
	3.0	18	65	do	
BAP(3.0)+NAA	0.5	20	65	Milky, Greenish	+
	1.0	21	65	do	+
	1.5	20	70	do	+
	2.0	18	75	do	++
	2.5	19	70	do	++
	3.0	19	70	do	++
BAP(3.0)+IAA	0.5	19	55	Milky, Greenish	+
	1.0	20	60	do	+
	1.5	21	60	do	+
	2.0	20	70	do	++
	2.5	21	70	do	++
	3.0	20	70	do	++

BAP(3.0)+2,4-D	**0.5**	**20**	**60**	**Milky, Greenish**	**+**
	1.0	**20**	**70**	**do**	**+++**
	1.5	**20**	**80**	**do**	**+++++**
	2.0	**18**	**90**	**do**	**+++++**
	2.5	**19**	**80**	**do**	**+++**
	3.0	**19**	**80**	**do**	**+++**

Table.4. Metabolite contents in sub- cultured callus derived from internodal explants of *L. inermis*

Days after inoculation	Starch (mg per g DW)	Total soluble sugars (mg per g DW)	Reducing sugars (mg per g DW)	Total phenol (mg per g DW)	Total soluble proteins (mg per g DW)
0	**35.3 ± 1.3**	**38.2 ± 1.2**	**20.3 ± 1.3**	**53.3 ± 2.4**	**2.1 ± 0.3**
05	**32.2 ± 1.1**	**41.4 ± 1.4**	**18.3 ± 1.1**	**57.1 ± 2.6**	**2.4 ± 0.5**
10	**30.1 ± 1.2**	**45.2 ± 1.3**	**17.3 ± 1.5**	**60.3 ± 2.9**	**2.9 ± 0.6**
15	**28.0 ± 1.1**	**60.3 ± 1.9**	**12.3 ± 1.4**	**63.1 ± 2.2**	**3.6 ± 0.4**
20	**26.2 ± 1.2**	**65.2 ± 1.8**	**15.1 ± 1.2**	**65.4 ± 2.5**	**4.1 ± 0.3**
25	**24.3 ± 1.5**	**68.1 ± 1.5**	**17.3 ± 1.4**	**66.1 ± 2.8**	**4.4 ± 0.4**

Table. 5. Activity of various enzymes in sub- cultured callus derived from internodal explants of *L. inermis*

Days after inoculation	α-amylase Starch hydrolysed (mg/g DW)	Acid -invertase Reducing sugars released (u mol/h/mg protein)	Acid –peroxidase Reducing sugars released (u mol/h/mg protein)
0	**16.1 ± 1.3**	**4.50 ± 0.3**	**4.18 ± 0.5**
5	**17.2 ± 1.1**	**4.13 ± 0.4**	**3.77 ± 0.3**
10	**18.1 ± 1.3**	**3.71 ± 0.3**	**3.68 ± 0.5**
15	**22.0 ± 1.1**	**3.50 ± 0.4**	**3.53 ± 0.4**
20	**24.2 ± 1.2**	**2.59 ± 0.2**	**2.41 ± 0.2**
25	**25.3 ± 1.5**	**2.32 ± 0.3**	**2.25 ± 0.4**

Table . 6. Mineral elements from leaves of mature plant and *in vitro* raised plants of *L. inermis*

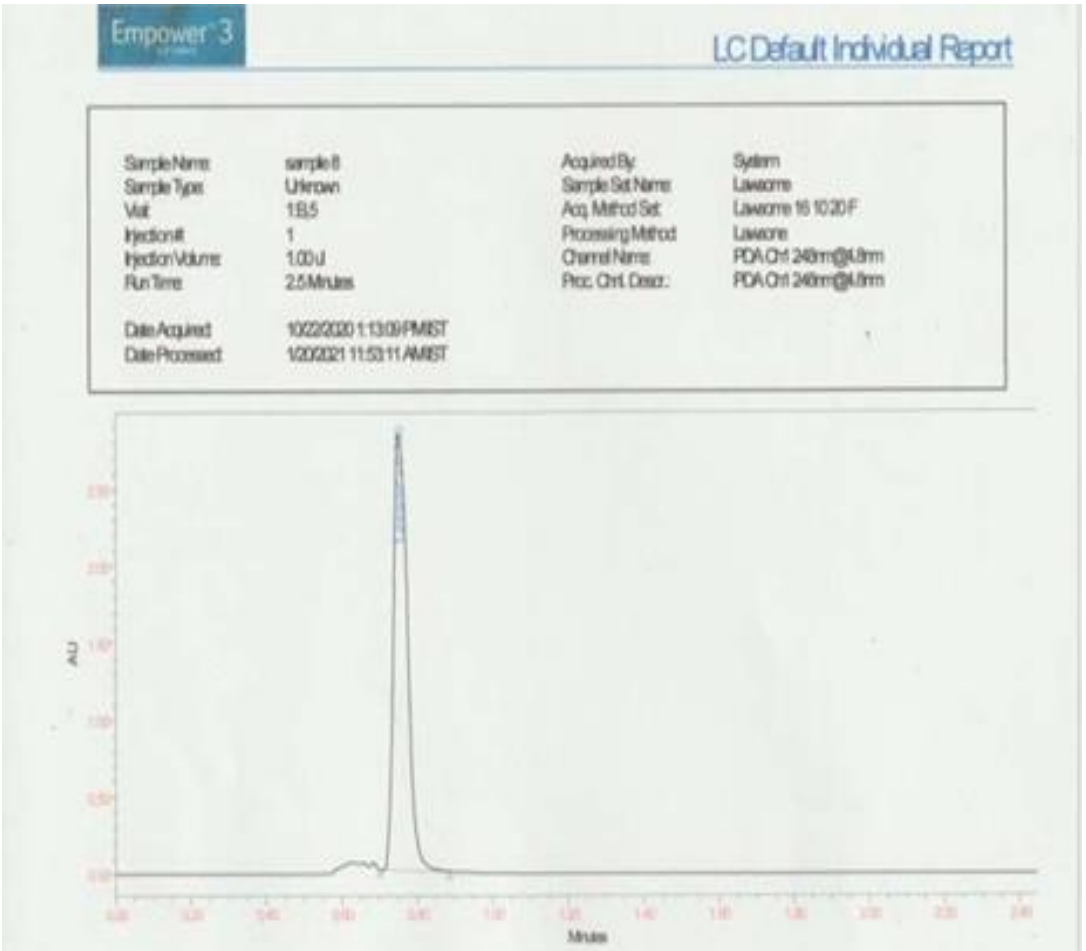
S. No.	Sample	**Nitrogen** (mg per g DW.)	**Phosphorus** (mg per g DW.)	**Potassium** (mg per g DW)
1	**Mother Plant**	**27.6**± 1.4	**5.1**± 0.4	**26.1**± 1.3
2	**Regenerated plant**	**31.5**± 1.2	**6.8**± 0.3	**29.3**± 1.6
3	**Callus**	**29.3**± 1.3	**5.9**± 0.5	**27.5**± 1.4

Table . 7. Lawsone quantification from leaves of mature plant and *in vitro* raised plants of *L. inermis*

S. No.	Sample	Peak area	Retention time	Lawsone content(µg/ mg)
1	**Mother Plant**	0.748	6834409	**0.0871**
2	**Regenerated plant**	0.748	9084893	**0.154**

Table.8 . Effect of additives on induction of lawsone in calli derived from leaf explants of *Lawsonia inermis*

Media	Additives (mg/l)				Area	Concentration (µg /mg)
	2,4-D	Casein Hydrosylate	Adenine sulphate	Yeast Extract		
MS	2.0	-	-	-	3411357	0.0281
MS	2.0	10	-	-	5017935	0.0352
MS	2.0	20	-	-	4742230	0.0331
MS	2.0	30	-	-	4004625	0.0303
MS	2.0	-	10	-	7380603	0.0675
MS	2.0	-	20	-	7996742	0.0715
MS	2.0	-	30	-	7260529	0.0824
MS	2.0	-	-	10	6471224	0.0413
MS	2.0	-	-	20	6865112	0.0489
MS	2.0	-	-	30	7260529	0.0535



Peak Name: Lawsone

	Peak Name	RT	Area
1	Lawsone	0.750	726529
Main			

Fig. 4.10. Lawsone content of callus under Adenine sulphate treatment (30 mg /l).

Reported by User: System
Report Method: LC Default Individual Report
Report Method ID: 5728 5728
Page: 1 of 1

Project Name: Phenolics
Date Printed:
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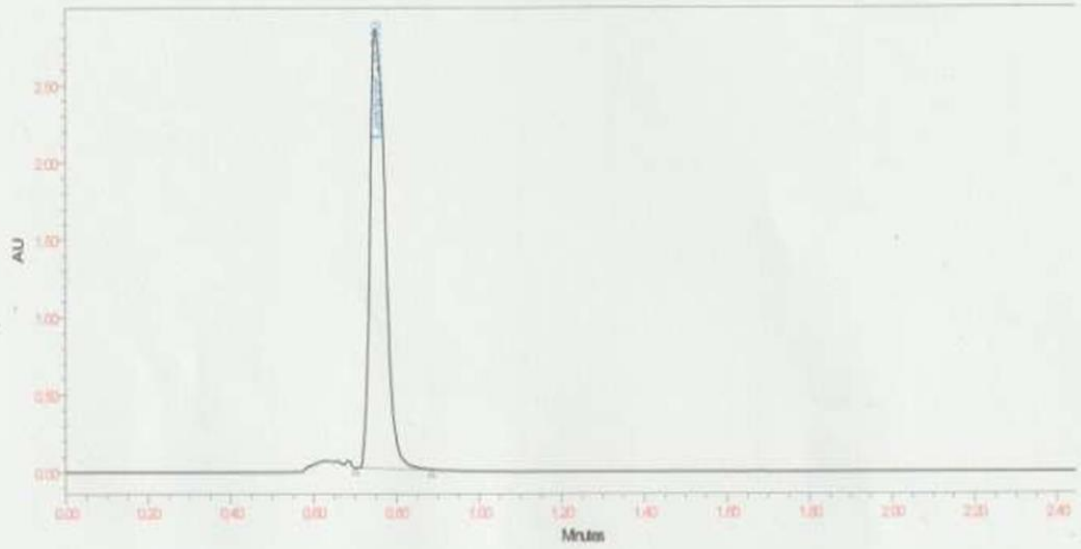
Sample Name:	sample 8	Acquired By:	System
Sample Type:	Unknown	Sample Set Name:	Lawsone
Vial:	1:B,5	Acq. Method Set:	Lawsone 16 10 20 F
Injection#:	1	Processing Method:	Lawsone
Injection Volume:	1.00 ul	Channel Name:	PDA Ch1 248nm@4.8nm
Run Time:	2.5 Minutes	Proc. Chnl. Descr.:	PDA Ch1 248nm@4.8nm
Date Acquired:	1/22/2020 1:13:09 PM IST		
Date Processed:	1/20/2021 11:53:11 AM IST		



Peak Name: Lawsone

	Peak Name	RT	Area
1	Lawsone	0.750	7260529
Mean			

Fig. 4.10. Lawsone content of callus under Adenine sulphate treatment (30 mg /l).

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Report Method: LC Default Individual Report
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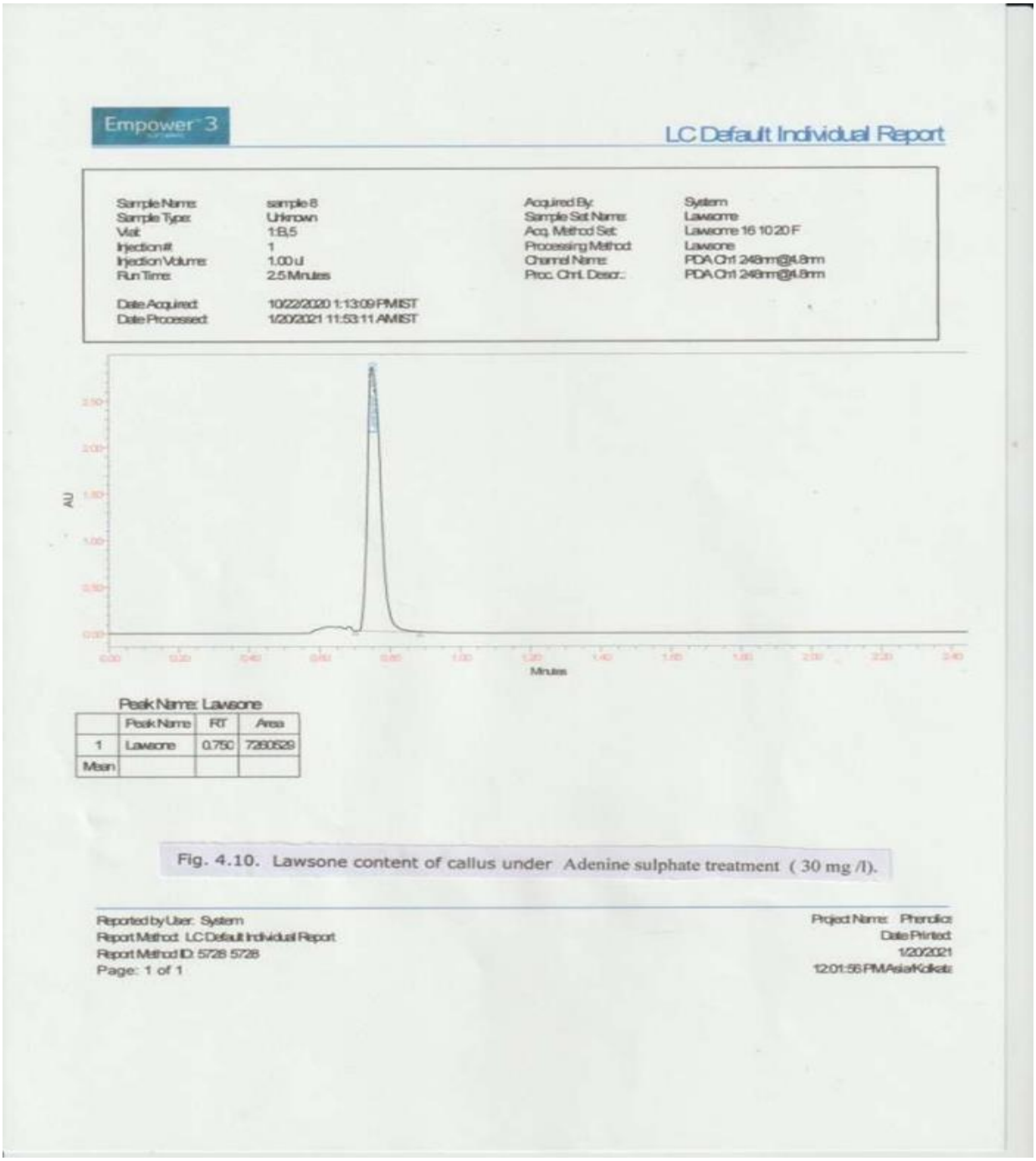

Fig. 4.10. Lawsone content of callus under Adenine sulphate treatment (30 mg /l).

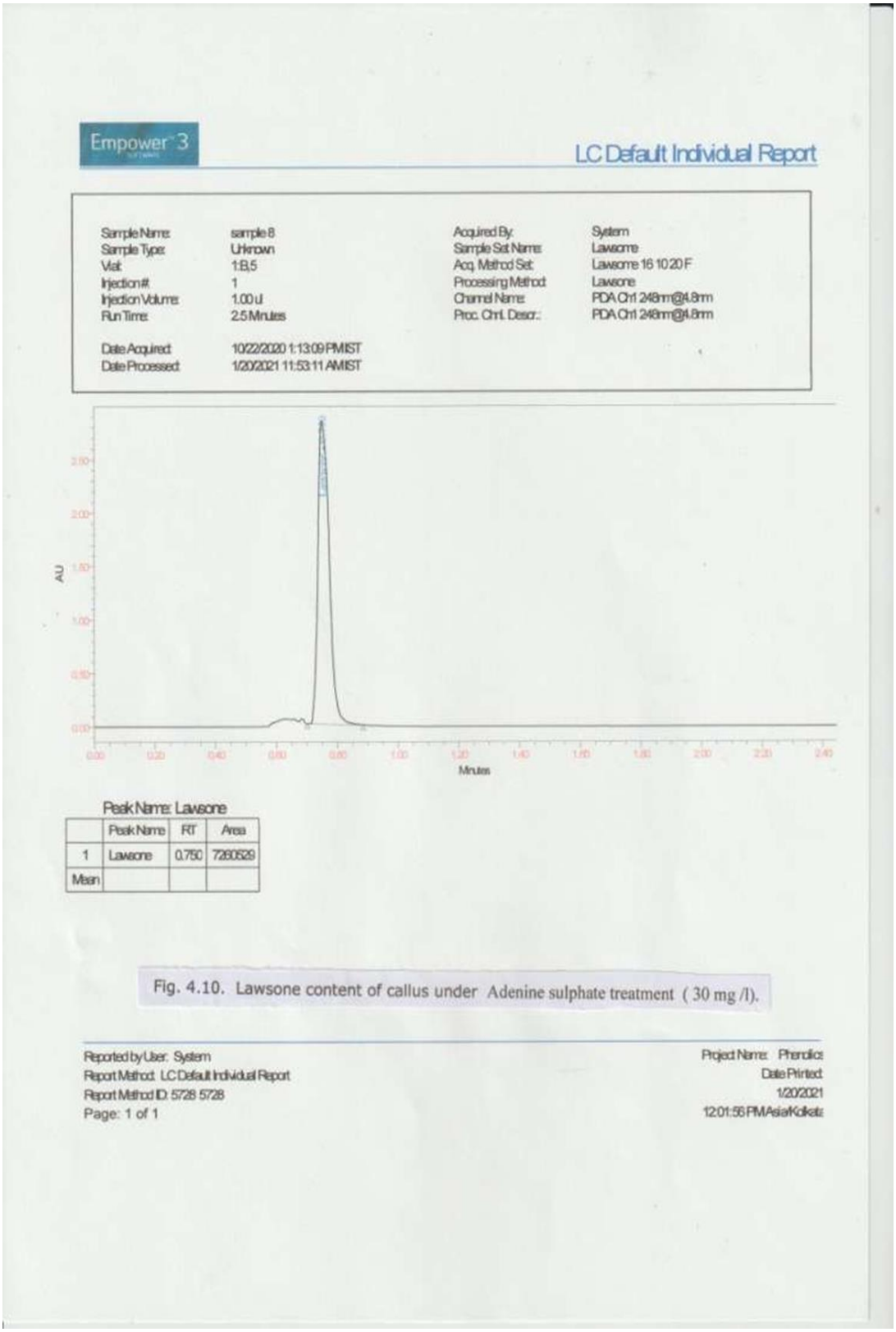

Fig. 4.10. Lawsone content of callus under Adenine sulphate treatment (30 mg /l).

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